

Differential reactive oxygen and nitrogen production and clearance of *Salmonella* serovars by chicken and mouse macrophages

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Abstract

The objective of the present study was to compare the uptake and killing of *Salmonella* serovars by murine and avian macrophage cell lines. We used *Salmonella enterica* serovars Enteritidis (SE338) and Typhimurium (SR11) for this study. Uptake of green fluorescent protein-labeled bacteria was measured using flow cytometry. Cell sorting and plating of viable infected macrophages demonstrated that bacterial clearance was significantly better with J774A.1 compared with HD11 cells. HD11 cells produced significantly higher amounts of nitric oxide (NO) than J774A.1 cells upon infection with SE338 and SR11, whereas J774A.1 cells exhibited greater superoxide production with SR11. Treatment of HD11 cells with recombinant chicken interferon gamma in the absence of bacteria enhanced NO production but did not induce increased levels synergistically with bacteria. Interferon treatment did not influence phagocytosis or increase killing by HD11 cells. Published by Elsevier Ltd.

Keywords: *Salmonella*; Chicken macrophages; Mouse macrophages; J774A.1; HD11; Nitric oxide; Superoxide; Flow cytometry

1. Introduction

Salmonella enterica are facultative intracellular bacteria of which serovar Typhimurium (ST) and serovar Enteritidis (SE) have a broad host range, including the capacity to cause human infections. Infection of humans by these organisms usually occurs by food-borne transmission. Human infection with SE is primarily caused by consumption of contaminated raw or partially cooked shell eggs while ST is implicated in contamination of chicken

meat and of a variety of other foods [1–6]. When ingested by humans, SE and ST initially infect intestinal mucosal cells causing a transient diarrhea but rarely become systemic. In avian and murine hosts the infections can become systemic. In the mouse, this occurs as a result of bacteria translocating across the mucosa to sub-mucosal tissues including Peyer's patch lymphoid structures [7]. There, *Salmonellae* are taken up by phagocytes including monocytes and macrophages [8]. As facultative intracellular pathogens, they are able to persist within these cells and become disseminated to spleen, liver and other tissues, as monocytes circulate in blood and lymphatics [9]. In susceptible mice, systemic ST infection with a virulent strain

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results in a typhoid-like illness that can result either in the death of the host or clearance of the infection with a resulting sterile immunity, usually within 1–3 weeks [10]. The immune response in mice involves innate, cell-mediated and antibody components [7,8,10,11]. In chickens infected orally with SE, a similar colonization of organs occurs and includes the reproductive organs of hens leading to contamination of shell eggs [12]. In contrast to mice, colonization of the chicken gastrointestinal tract and tissues by SE often occurs without signs of overt clinical symptoms; furthermore, egg production of hens is not affected by SE infection [2]. Infection can persist for up to 18 weeks in laying hens. Experimental infection of chickens with SE results in both cell-mediated and antibody responses that are ultimately not effective in clearing the infection [13,14]. Induction of measurable immune protection with live, attenuated and heat-killed vaccines, suggests that in the absence of vaccination, the immune response has limited effectiveness [15]. This species-specific pathogenesis may be due to a variety of factors influencing differential host–pathogen interaction, including differences in interaction with components of cell-mediated and innate immunity including phagocytes, which play a central role in pathogenesis in both mouse and chicken. The ability of *Salmonellae* to survive within host cells is essential for the establishment of systemic infection [16]. With respect to the mouse model, *Salmonella* pathogenicity island encoded type III secretion system genes play a major role in host cell invasion and survival in macrophages in both ST and SE [17,18]. Activation of macrophages by inflammatory mediators such as interferon gamma and the importance of reactive oxygen and nitric oxide (NO) in killing of *Salmonellae* by primary mouse macrophages and macrophage cell lines, including J774A.1 is well established [7,8,10]. The in vitro interaction of avian phagocytic cells with *S. enterica* serovars has more recently been investigated. The activation by IL-2 of chicken heterophils, professional phagocytes analogous to mammalian polymorphonuclear cells, induces IL-8 and IL-18 mRNA following phagocytosis of SE [19]. Enhanced heterophil activation is associated with increased resistance and with cytokine mRNA expression [20]. In studies with the species-specific *S. enterica*, serovar Pullorum, splenic macrophages were found to play a role in persistent infection by harboring bacteria for 40 weeks after infection [21]. Macrophages from *Salmonella*-resistant chickens

were also found to kill serovar Gallinarum more efficiently than macrophages from *Salmonella*-susceptible chickens, suggesting an important role for macrophages in this genetically based resistance [22]. In a recent study, we investigated the effect of recombinant chicken interferon- γ (rchIFN- γ) on the infection of primary chicken macrophages isolated from peripheral blood with ST and SE. ST showed an increased ability to survive in primary macrophages and interferon treatment caused increased cellular necrosis in combination with infection [23]. The ability to conduct these studies was inhibited by difficulty in obtaining and maintaining consistent populations of primary macrophages in large enough numbers. The use of a chicken macrophage cell line provided a means to overcome this problem. In previous studies, the avian monocyte–macrophage cell line HD11 showed increased bactericidal activity in vitro as well as enhanced production of cytokines and NO following exposure to CpG oligodeoxynucleotides [24,25] and was therefore a candidate for this purpose.

While there have been comparisons of *Salmonella* serovars in mouse and human macrophage cell lines [26] as well as in vivo comparisons in both mouse and chicken experimental infection [27], no studies have specifically focused on comparing SE and ST interaction with mouse and chicken macrophages. In this study, we have conducted in vitro infection studies with mouse and chicken macrophage cell lines focused on cellular measurement of uptake and survival of ST and SE using flow cytometry and cell sorting. The use of green fluorescent protein (GFP)-labeled SE and ST in combination with flow cytometry has proven to be a valuable approach, allowing for a more quantitative analysis of the dynamics of macrophage–bacteria interactions in vitro [24,28]. NO and superoxide production was also measured. In addition, the effect of recombinant IFN- γ on these parameters in chicken macrophages was also assessed. The results will shed light on the unique features of *S. enterica* infection in mice and chickens that dictate the differential response of mouse and chicken macrophages following infection.

2. Materials and methods

2.1. Cell lines

The murine monocytic cell line J774 A.1 (American type culture collection, Rockville, MD, USA)

and HD11 chicken macrophage-like cells [29] were maintained in RPMI 1640 tissue culture medium (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated (56 °C, 1 h) fetal bovine serum (FBS) (Hyclone) as previously described [24,28]. For in vitro infection experiments, cells from overnight culture flasks were washed twice by centrifugation (500g, 5 min) with RPMI 1640 tissue culture medium with 10% FBS without antibiotics and re-suspended in the same medium at a concentration of 5×10^5 cells/ml.

2.2. In vitro infection of macrophages

GFP-labeled SE (SE338, phage type 4) and ST (SR11) used in our study were developed and maintained as previously described [24,30]. These strains are infective in mice (SR11) and chickens (SE338). SE338 was isolated from raw egg implicated in a human infection. Overnight cultures were set up in Luria broth at 37 °C. Cultures were washed twice with sterile phosphate buffered saline (PBS) re-suspended in PBS and adjusted to an OD⁵⁵⁰ of 0.5. This provided an approximate count of 10^8 CFU/ml. In vitro macrophage infection experiments were carried out as previously described [24]. Bacteria were added to macrophage suspension at ratio of 50–100 bacteria:1 macrophage. These suspensions were incubated at 37 °C and 5% CO₂ for approximately 1 h in RPMI 1640 with 10% FBS without antibiotics. After the initial uptake, cells were washed twice with RPMI 1640 with FBS containing gentamicin (GIBCO, Grand Island, NY, USA) at 10 µg/ml and re-suspended in the same medium. These cells were analyzed for uptake of fluorescent bacteria by flow cytometry. Bacterial killing by macrophages was assessed by cell sorting of the desired number of viable infected macrophages as previously described [24,28]. After cell sorting, the remaining sample was centrifuged and the supernatant collected and frozen at –20 °C for NO analysis.

2.3. NO analysis

Aliquots of 50 µl supernatants were mixed with 50 µl Griess reagent (Sigma, St. Louis, MO, USA) and incubated at room temperature for 10 min and were read in a ELISA plate reader at 550 nm. The amount of NO produced was calculated by comparing with a standard curve produced by using 0–200 nmoles of NaNO₂.

2.4. Superoxide analysis

Superoxide anion (O₂[–]) production in response to SE and ST infections was determined as superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C. Macrophages (J774A.1 and HD11) were infected with SE or ST, in the presence and absence of SOD (155 units/ml) (Sigma) for 90 min. After the bacterial uptake, cells were washed and re-suspended in HBSS containing 10% FBS and gentamicin (10 µg/ml). After washing the cells to remove non-phagocytosed bacteria, the same amount of SOD was added to cells that were previously treated with SOD. Cells (50 µl aliquots) were analyzed for O₂[–] immediately after the bacterial uptake and 24 h post-infection by adding 10 µl cytochrome C (160 µM) and monitoring the change in absorbance at 550 nm in a 96-well plate reader. Data are expressed as nmoles O₂[–]/10⁶ cells which were calculated by the molar extinction coefficient of $E_{550} = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$.

2.5. Treatment of macrophages with rchIFN-γ

rchIFN-γ was produced in transformed COS cells as previously described [31]. The optimal amount of rchIFN-γ was determined by titration using NO production and MHC class II expression as endpoints. A 1:50 v/v dilution induced maximal NO production and MHC class II expression. As a control, culture supernatant from non-transformed COS cells was used. HD11 cells were incubated in the presence of a 1:50 v/v dilution of rchIFN-γ or control supernatant for 18 h, followed by washing to remove IFN. Cells were then infected with SE or ST strains as described above.

2.6. Impact of SOD on bacterial uptake and killing

To determine the role of O₂[–] in bacterial killing, macrophages were pre-treated with SOD (155 units/ml) for 2 h. After the bacterial uptake, cells were washed and re-suspended in RPMI containing the same concentration of SOD. Aliquots were taken at baseline and 24 h post-infection to assess bactericidal activity. Data are presented as CFU/200 viable macrophages.

2.7. Statistical analysis

Data pertaining to NO, O₂[–] and bacterial killing were analyzed by conducting one-way analysis of

variance followed by Student–Newman–Keuls test or *t*-test. These statistical analyses were done using Sigma Stat v2.01. Differences between the treatment groups were considered statistically significant at $P < 0.05$.

3. Results

3.1. Uptake and killing of SE and ST by J774 and HD11 cells

Representative flow cytometry histograms of J774A.1 and HD11 cells infected with GFP-labeled SE and ST (Fig. 1) illustrate the method used to determine the percent infected macrophages, and the mean relative fluorescence intensity of infected cells at baseline (time 0) and 24 h after infection. Results of 12 experiments were used to compile means for these values (Fig. 2). The percentage of J774A.1 and HD11 cells containing intracellular ST and SE was similar when exposed at the same MOI (Fig. 2A). The mean fluorescence intensity, however, indicated that J774A.1 mouse macrophages phagocytized more bacteria per cell ($p < 0.05$) (Fig. 2B). The lower mean fluorescence of ST infected cells is due to the lesser fluorescence intensity per bacterium of GFP-ST. Comparing results of lysis and plating of sorted GFP positive macrophages with the flow cytometry results indicated that there were major differences in SE and ST clearance by J774A.1 and HD11 cells (Fig. 3). Greater numbers of ST, relative to SE, were recovered from both macrophage lines at baseline and 24 h. Comparing the two macrophage lines, significantly fewer SE were recovered from J774A.1 at baseline and 24 h ($P < 0.05$) (Fig. 3). Both SE and ST colony forming units decreased more between baseline and 24 h in J774A.1 cells relative to HD11. Thus, despite the fact that there are more bacteria per cell in J774A.1, fewer viable bacteria per cell were recovered, indicating greater bacterial killing by J774A.1 versus HD11.

3.2. NO and superoxide production by J774 and HD11 cells in response to SE and ST infection

HD11 cells produced significantly more NO in response to SE and ST infections than did J774A.1 cells at 24 h ($p < 0.05$). There was no significant difference in the amount of NO produced between SE and ST infections within each macrophage cell line (Fig. 4A). Superoxide production was significantly

higher in J774A.1 cells 24 h after infection with ST. HD11 showed a significant superoxide increase with ST as well, but this increase was less than that seen with J774A.1 and ST (Fig. 4B). SE did not induce significant increases in superoxide in either cell line.

3.3. Effect of *rchIFN-γ* on HD11–salmonella interactions

The less effective anti-bacterial response of HD11, relative to J774A.1, led to an investigation of the effect of *rchIFN-γ* on phagocytosis, bactericidal activity and NO production in HD11. Incubation with *rchIFN-γ* for 18 h resulted in increased granularity (as indicated by side light scattering, data not shown) characteristic of activated macrophages. Macrophages were washed to remove *rchIFN-γ* and infected with SE or ST. Non-infected control cultures were also included. Non-infected control cultures that had been incubated with *rchIFN-γ* showed an increase in NO production relative to control *IFN-γ* supernatants at 24 h indicating the activating effects of *rchIFN-γ* (Fig. 5). NO levels in culture supernatants from both SE and ST-infected HD11 cells were greatly increased above non-infected cells, whether or not they had been activated by *rchIFN-γ* (Fig. 5). *rchIFN-γ* did not result in a significant increase in phagocytosis of either SE or ST at baseline as determined by flow cytometry (data not shown). Bacterial recovery at baseline and 24 h reflected this finding with no significant difference between *rchIFN-γ*-treated HD11 and controls (Fig. 6). Since there was no significant effect of *rchIFN-γ* on killing of bacteria, its effect on superoxide production was not determined.

3.4. Effect of SOD on Salmonella–macrophage interactions

Because ST infection induced superoxide production, we investigated the effects of SOD on bactericidal activity of J774A.1 and HD11. SOD had no effect on survival of SE in either macrophage cell line (Fig. 7). SOD treatment, however, resulted in increased survival of ST in J774A.1 cells at 24 h. SOD had no effect on survival of ST in HD11 cells.

4. Discussion

In this study, we employed an approach similar to the one used to investigate the interactions of mouse and human macrophage cell lines with *Salmonella typhi* and ST [26]. The rationale for this approach

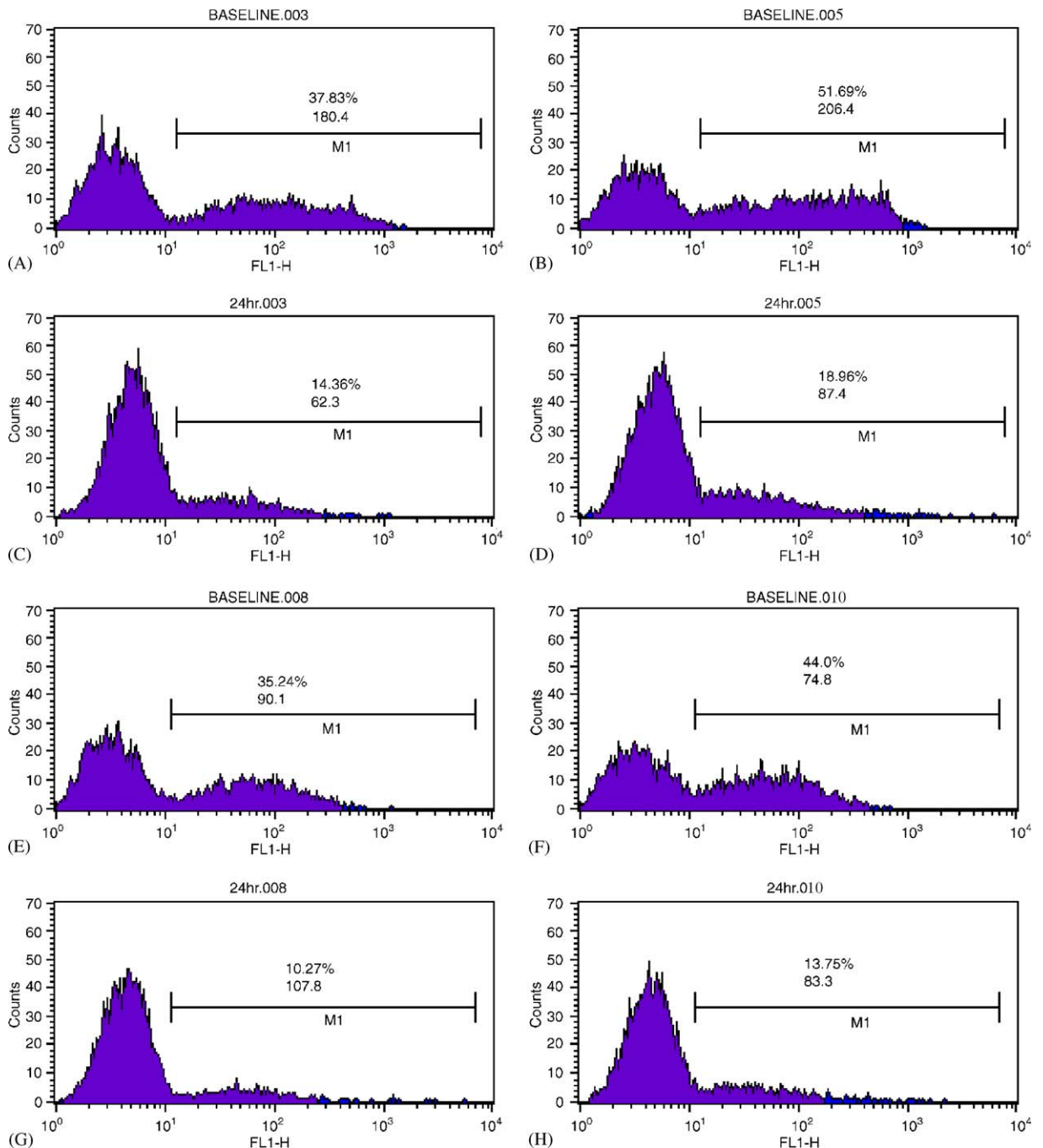


Fig. 1. Phagocytosis of GFP-labeled *Salmonella enterica* serovar Enteritidis (A,C,E,G) and *Salmonella enterica* serovar Typhimurium (B,D,F,H) by J774A.1 (A–D) and HD11 (E–G) macrophage cell lines as measured by flow cytometry. The X-axis of each histogram represents intensity of GFP fluorescence (log scale), the Y-axis represents cell number. Analysis for each cell line and serovar was done at 0 (baseline) and 24 h after infection. The percentage of cells within the positive region (M1) is shown along with the mean fluorescence intensity for positive cells.

lies in the importance of macrophages in resistance to infection in vivo, as demonstrated in the mouse model with ST and SE [7,8,10]. Facultative

intracellular bacteria such as *Salmonella* are capable of surviving within bacterially modified macrophage spacious vacuoles [32]. The ability of virulent

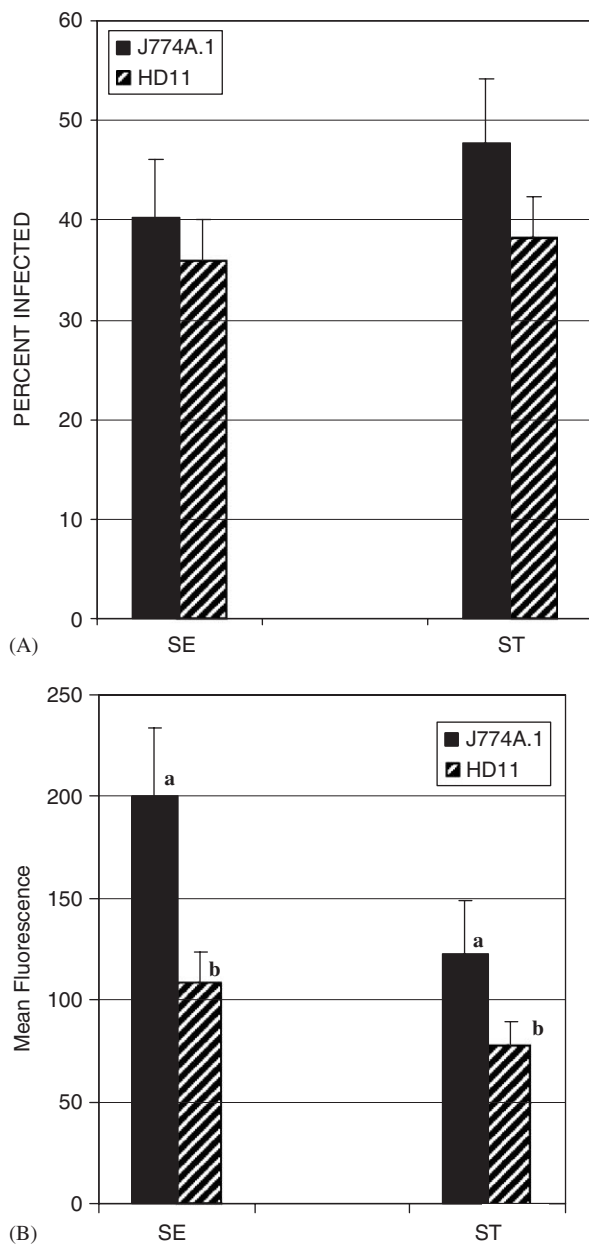


Fig. 2. (A) Percentage of J774A.1 and HD11 cells infected with GFP-labeled *Salmonella enterica* serovar Enteritidis (SE) or *Salmonella enterica* serovar Typhimurium (ST) at baseline. Each bar represents mean \pm SEM for 6–12 independent experiments. Percent infected cells was determined by analysis of 5000 viable cells in each experiment. No significant differences were observed. (B) Mean fluorescence intensity of J774A.1 and HD11 cells infected with GFP-labeled *Salmonella enterica* serovar Enteritidis (SE) or *Salmonella enterica* ST at baseline. Each bar represents mean \pm SEM for 6–12 independent experiments. Mean fluorescence is based on analysis of 5000 viable cells in each experiment. Different letters on the bars indicate a significant difference between J774 A.1 and HD11 cells at $p < 0.05$.

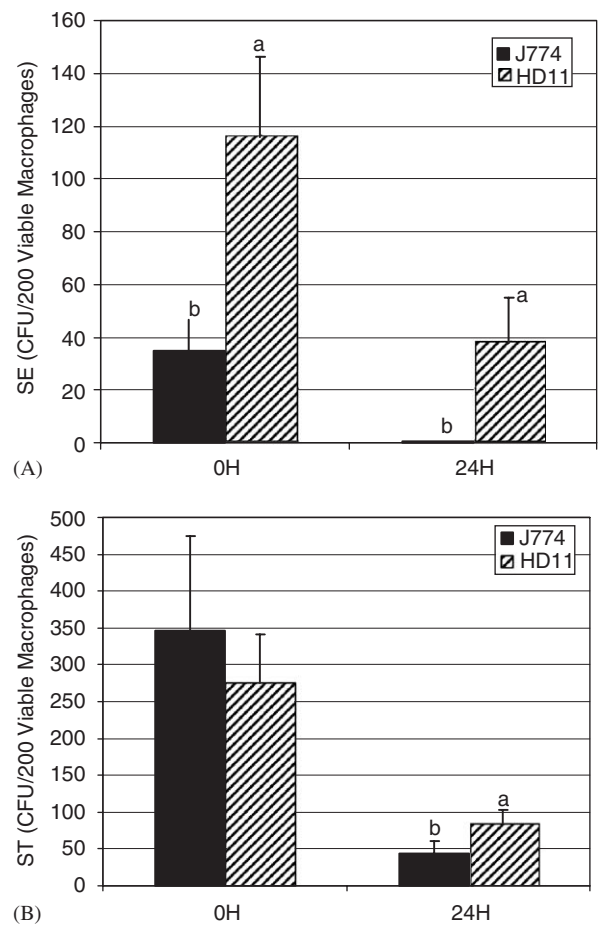


Fig. 3. *Salmonella enterica* serovar Enteritidis (SE) (A) and *Salmonella enterica* serovar Typhimurium (ST) (B) survival in J774 and HD11 cells at baseline (0H) and 24 h post-infection. Each bar represents mean \pm SEM for 6–12 independent experiments and each data point is an average of triplicates. Data are presented as CFU/200 viable infected macrophages. Different letters on the bars indicate a significant difference between J774 A.1 and HD11 cells at 0 and/or 24 h ($p < 0.05$).

Salmonella to cause systemic infection in mice is dependent upon this capability [33]. A type III secretion system encoded by *Salmonella* pathogenicity island 2 (SPI 2) is essential for evasion of reactive oxygen species within phagosomes [34,35]. Through antigen processing and the release of soluble inflammatory mediators, macrophages are also involved in induction of the adaptive immune response and, when activated by cytokines, are instrumental in clearing of the infection in mice [36,37]. The role of macrophages in *S. enterica* infection of chickens is not as clearly defined, and may be different, given the fact that experimental infection in laying hens can persist for many weeks,

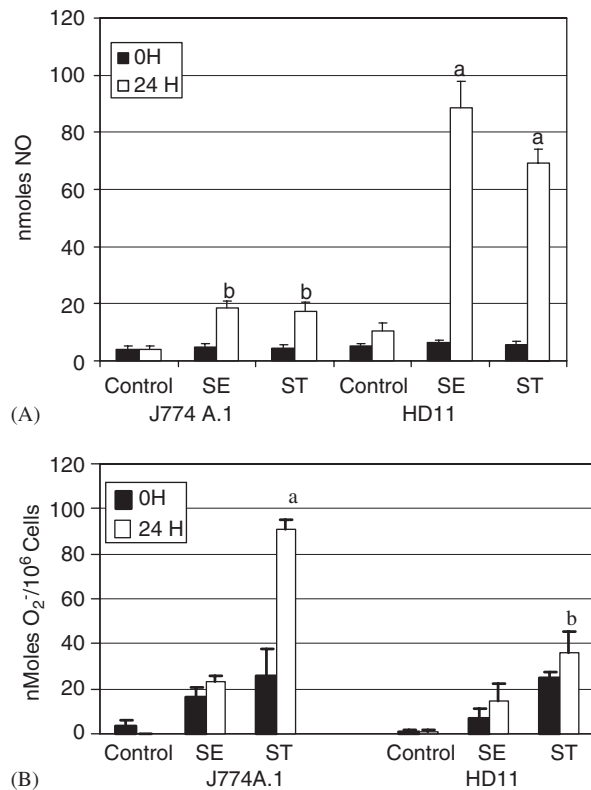


Fig. 4. (A) Nitric oxide (NO) produced by J774 and HD11 cells at 0 (baseline) and 24h post *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Typhimurium (ST) infections. Each bar represents means \pm SEM from 6–8 experiments. Different letters on the bars indicate a significant difference between J774 A.1 and HD11 cells at $p < 0.05$. (B) Superoxide (O₂⁻) produced in response to *Salmonella enterica* SE and *Salmonella enterica* ST infection by J774 and HD11 cells at baseline (0h) and 24h post-infection. Each bar represents mean \pm SEM from 4–8 independent experiments and each data point is an average of quadruplicate measurements. Data are presented as nmoles O₂⁻ produced by 10⁶ cells. Different letters on the bars indicate a significant difference between J774 A.1 and HD11 cells at $p < 0.05$.

with little overt harm to the host [2,30]. In contrast, the course of infection with virulent salmonellae in susceptible mice may be more rapid, resulting in either clearance of the infection or death of the host [10,38,39].

In genetically resistant mice, the occurrence of a persistent carrier-like infection in macrophages found in mesenteric lymph nodes, has recently been described [40]. Persistence of *S. enterica* serovar Pullorum in chickens is also associated with survival within splenic macrophages [21]. Primary macrophages isolated from chickens genetically resistant to *S. enterica* demonstrated increased respiratory

burst in response to and increased killing of serovar Gallinarum versus macrophages from a susceptible strain, suggesting that macrophage function played a significant role in the resistant phenotype [22]. These same studies indicated that killing of serovars Typhimurium and Enteritidis was not significantly different between resistant and susceptible primary macrophages, nor was the respiratory burst in macrophages from adult birds.

We have recently reported results of similar studies conducted with normal primary macrophages isolated from adult chicken peripheral blood [23]. Those studies investigated parameters similar to the present work, including determination of differences between ST and SE in their interactions with primary macrophages, and the effects of rhIFN- γ . Similar to the current results with HD11 cells, there was no difference in phagocytosis between ST and SE, and rhIFN- γ did not affect phagocytosis. We also found that, as in the current study, there was enhanced survival of ST versus SE in primary macrophages. In contrast to HD11, rhIFN- γ increased bactericidal activity of primary macrophages, and NO production was enhanced in infected cells 24h after treatment with rhIFN- γ . This comparison suggests that there are both similarities and differences between the responses of HD11 and primary macrophages. There may be questions regarding the in vivo relevance of data obtained with transformed cells such as HD11; however, significant problems also exist with isolated primary cells. These problems revolve around obtaining sufficient numbers of uniform, viable cells that are consistent between lots. Indeed, as we and others have reported [22,23], viability of primary macrophages declines significantly within 24h, even in non-infected cells. With respect to in vivo relevance, results that are similar in primary macrophages and tissue culture lines are probably most significant. The current studies were directed at further investigating those differences and drawing comparisons with the mouse model using macrophage cell lines from chickens and mice, where larger numbers of highly uniform cells that remain viable over time could be obtained.

Although flow cytometry data and the colony counts from sorted infected cells both indicated that SE and ST were cleared more efficiently by mouse J774A.1 cells than by avian HD11 cells, colony counts may have understated the difference. Compared with HD11, the colony counts were lower in J774A.1 cells at baseline with SE and higher with

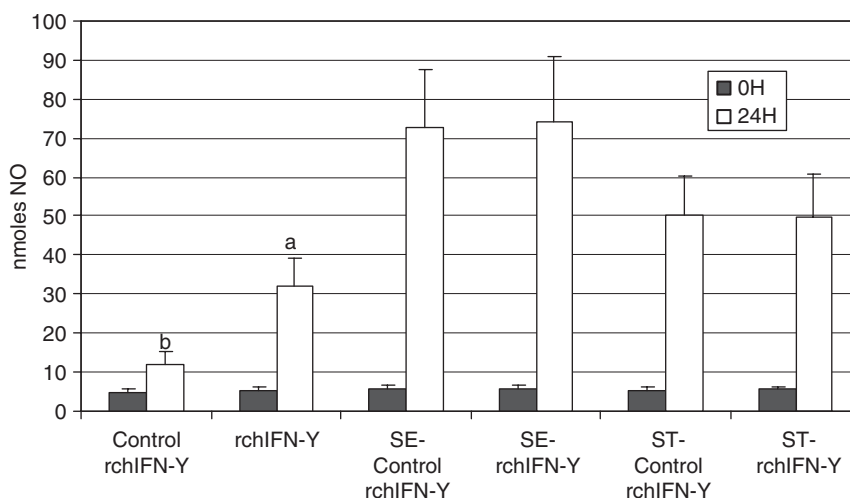


Fig. 5. Nitric oxide (NO) production by HD11 cells in response to *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Typhimurium (ST) infection with and without chicken γ -interferon (rchIFN- γ) at baseline (0 h) and 24 h post-infection. Each bar represents mean \pm SEM for seven independent experiments. Data are presented as nmoles NO using 50 μ l supernatant. Different letters on the bars indicate a significant difference between control rchIFN- γ (supernatant from non-transformed COS cells) and rchIFN- γ -treated HD11 cells at $p < 0.05$.

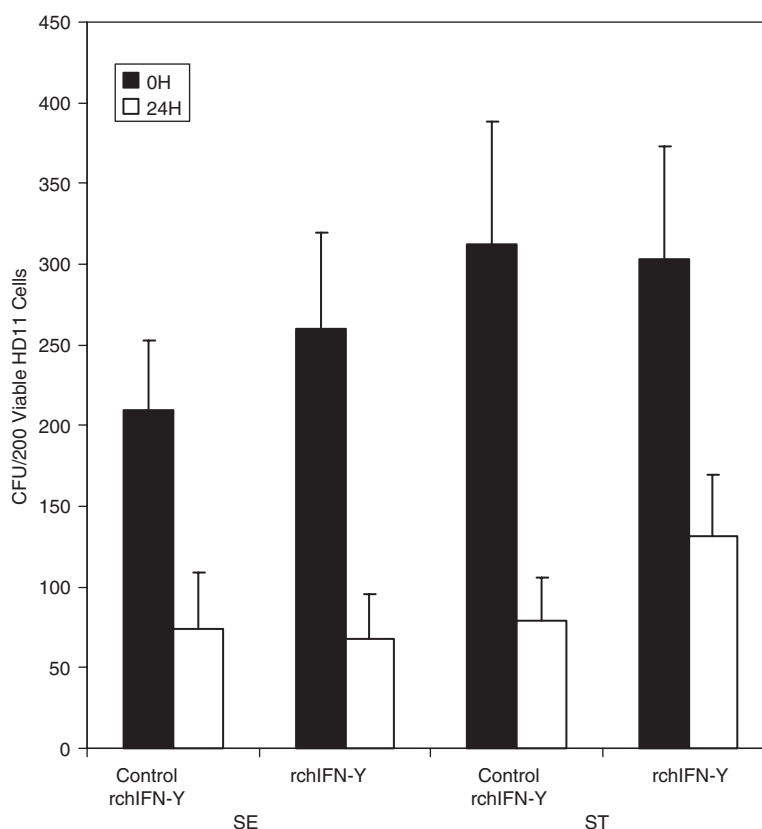


Fig. 6. Impact of rchIFN- γ on *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Typhimurium (ST) survival within HD11 cells at baseline (0 h) and 24 h post-infection. Each bar represents mean \pm SEM for seven independent experiments and each data point is an average of triplicates. Data are presented as CFU/200 viable infected macrophages. No statistically significant differences were seen between rchIFN- γ -treated cells and controls.

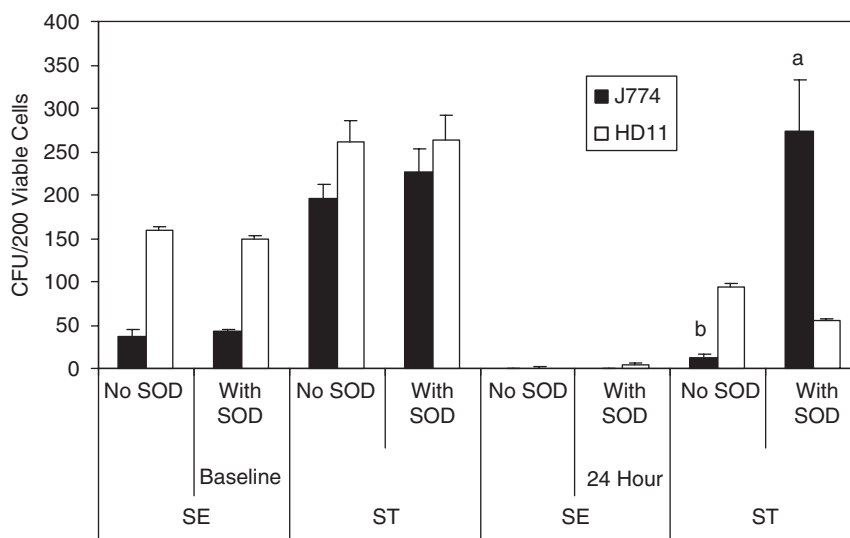


Fig. 7. Impact of superoxide dismutase (SOD) on *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Typhimurium (ST) survival in J774 and HD11 cells at baseline (0h) and 24h post-infection. Each bar represents mean \pm SEM for six independent experiments and each data point is an average of triplicates. Data are presented as CFU/200 viable infected macrophages. Different letters on the bars indicate a significant difference between SOD-treated and untreated J774A.1 cells at $p < 0.05$.

ST. In the absence of flow cytometry analysis, the baseline colony counts might be interpreted as indicating less infection of J774A.1 by SE; however, flow cytometry indicated that the percent of SE-infected cells in these cell lines was similar. The reduced SE colony counts at 0h in J774A.1 are indicative of more dynamic bactericidal activity, relative to HD11. Further amplifying this effect, the mean fluorescence per macrophage at 0h was also higher in J774A.1, indicating phagocytosis and killing of more bacteria/cell on average. With respect to ST, higher colony counts in both cell lines at 0h indicated a greater resistance to macrophage bactericidal activity by ST. The higher recovery of ST versus SE at 24h confirmed this trend. If these in vitro interactions reflect events in vivo, then increased survival of *S. enterica* within avian macrophages may allow for extensive dissemination and long-term colonization of chicken tissues, including ovaries, which would have implications for egg contamination.

Attempts to augment *Salmonella* killing by HD11 using rchIFN- γ activation did not significantly enhance phagocytosis or killing of ST or SE. rchIFN- γ induced significant NO production in non-infected HD11 cells and also resulted in increased cell granularity based on side light scatter as determined by flow cytometry, indicating that the cells responded to rchIFN- γ treatment based on

these two parameters. However, there was no increase in NO production above levels induced by ST and SE alone in the presence of control IFN- γ , indicating the response to rchIFN- γ and the response to bacteria are not synergistic at the levels tested. As reported from gene expression studies in resistant and susceptible chickens infected with SE, the IFN- γ gene was expressed at lower levels in susceptible birds, suggesting a possible role for this cytokine in maintenance of the carrier state in resistant birds [41] possibly through a macrophage-dependent pathway. Similarly, our previous studies with primary peripheral blood macrophages [23] indicate that IFN- γ treatment increases killing of SE and ST. This suggests that the response of HD11 to IFN- γ does not replicate that of primary macrophages with respect to enhanced bactericidal activity. A more definitive assessment of the role of IFN- γ in vivo could be accomplished by anti-IFN- γ inactivation, in a manner analogous to studies in the mouse [40].

Well known and very efficient mechanisms for killing of intracellular bacteria exist in the mouse J774A.1 cell line, including, delivery of reactive oxygen species [42] and acidification of phagosomes [43]. Less is known about these and other bacterial killing mechanisms in chicken macrophage cell lines such as HD11; however, the intracellular killing of ST and SE reported here appears to be less effective

than in mouse macrophages. Induction of NO production appears to be more robust in HD11 than J774A.1, while superoxide production is more pronounced in J774A.1. This may indicate that superoxide production is a more bactericidal mechanism for killing of *Salmonella*, especially ST, in these cell lines. While SE and ST were equal in their ability to induce NO in both cell lines, only ST induced a significant superoxide response. Similar to our results, stimulation of oxidative burst by PMA and Zymosan has been reported to be greater in J774A.1 than HD11 [44]. In the same study, wide variation was found among *Salmonella* serovars in their ability to induce superoxide, with ST among the most potent; however, no SE strains were studied. In our study, superoxide was assessed by SOD-inhibitable cytochrome C reduction, which is an extracellular measure of this anion reflecting the activity of the plasma membrane NADPH oxidase [45]. Chadfield and Olsen were able to detect chemiluminescence by J774A.1 and HD11 cells in response to *Salmonella* serovars using lucigenin but not luminol as the probe [44]. It is known that Lucigenin cannot enter the cell, and therefore it reacts with the reactive oxygen in the extracellular environment whereas, luminol can enter the cells and help measure the intracellular production of reactive oxygen species. Thus, from our studies and those reported by Chadfield and Olsen, it appears that the respiratory burst induced by *Salmonella* serovars in both J774 and HD11 cell lines may be mediated extracellularly.

We observed significantly more superoxide induction in response to ST at 24 h post-infection compared with baseline time point, especially among J774 cells. Similarly, mouse peritoneal macrophages have been shown to produce 2.7-fold greater superoxide at 24 h compared to 2 h post-infection with *Mycobacterium tuberculosis* [46]. The kinetics of superoxide production appear to depend on the source of macrophages, type of stimulating agent and the sensitivity of the assay used to measure the reactive oxygen species.

In parallel to the increased superoxide production by J774A.1 cells in response to ST, SOD treatment significantly affected killing of ST by these cells, but not HD11, and had no effect on SE killing by either macrophage line. This is consistent with the enhanced induction of superoxide by ST in J774A.1. Similar to our observations, others have reported increased *Salmonella* recovery from murine hepatocytes that were treated with SOD [47]. Importance of

reactive oxygen species in *Salmonella* infections is further confirmed by the attenuation of virulence of *Salmonella choleraesuis* and ST by SOD mutants [48]. In the case of ST, the SPI-2 encoded ability to evade NADPH oxidase-mediated killing is an important virulence factor. The situation with SE is less clear. Even though virulent SE possesses SPI-2 genes, SE appears to be less efficient in induction of the superoxide response. As in our study, SE killing by mouse peritoneal macrophages was shown to be unaffected by SOD or catalase enzymes [49]. It has also been reported that chicken heterophils respond to SE with a relatively weak superoxide response compared to other serovars [50]. Reactive oxygen is an important mediator of tissue damage and inflammation in vivo. This could be related to the relatively weak inflammatory response to SE in chickens [14,51] which could account for a more benign, long-term relationship of SE in the avian host allowing for colonization of tissues including ovaries and resulting in egg contamination.

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